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L2: Entry 1 of 1 File: USPT Nov 23, 1999

US-PAT-NO: 5989169
DOCUMENT-IDENTIFIER: US 5989169 A

TITLE: .alpha.-amylase mutants

DATE-ISSUED: November 23, 1999

INVENTOR-INFORMATION:

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APPL-NO: 8/ 600908
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PARENT-CASE:
CROSS-REFERENCE TO RELATED APPLICATIONS This application is a continuation of PCT/DK96/00057 filed Feb. 5, 1996, which is incorporated herein by reference.

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FOREIGN PATENT DOCUMENTS

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WO 94/02597	February 1994	WOX

OTHER PUBLICATIONS

Machius et al. (Mar. 3, 1995) J. Mol. Biol. vol. 246, pp. 545-559.
MacGregor (1988) J. Protein Chemistry 7, 399-415.
Suzuki et al. (1989) J. Biol. Chem. 264, 18933-18938.

ART-UNIT: 162
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ABSTRACT:

The present invention relates to a method of constructing a variant of a parent Termamyl-like .alpha.-amylase, which variant has .alpha.-amylase activity and at least one altered property as compared to the parent .alpha.-amylase, comprising i) analyzing the structure of the parent Termamyl-like .alpha.-amylase to identify at least one amino acid residue or at least one structural part of the Termamyl-like .alpha.-amylase structure, which amino acid residue or structural part is believed to be of relevance for altering the property of the parent Termamyl-like .alpha.-amylase (as evaluated on the basis of structural or functional considerations), ii) constructing a Termamyl-like .alpha.-amylase variant, which as compared to the parent Termamyl-like .alpha.-amylase, has been modified in the amino acid residue or structural part identified in i) so as to alter the property, and iii) testing the resulting Termamyl-like .alpha.-amylase variant for the property in question.

23 Claims, 11 Drawing figures

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Borchert; Torben Vedel	Copenhagen N	N/A	N/A	DKX

US-CL-CURRENT: 435/201; 435/202, 435/203, 435/204, 435/252.3, 435/440, 536/23.2

CLAIMS:

We claim:
1. A method of producing a variant of a parent alpha-amylase having an altered property relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70% homologous to said sequence when homology is determined by GAP using default values for the GAP program (Genetic Computer Group, Version 7.3) penalties, said method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ ID-NO:13 depicted in Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) identifying in the three-dimensional structure obtained in step (a) at least one structural part of the parent wherein an alteration in said structural part is predicted to result in said altered property;
(c) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and
(d) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,
wherein the variant has alpha-amylase activity and has at least one altered property relative to the parent.
2. The method of claim 1, wherein the altered property is selected from the group consisting of substrate specificity, substrate binding, substrate cleavage pattern, temperature stability, pH dependence of enzymatic activity, pH dependence of stability, stability towards oxidation, Ca.sup.2+ -dependency and specific activity.
3. The method of claim 2, wherein the altered property is the calcium ion dependency and the structural part is selected from the group consisting of the C domain, the interface between the A and B domain, the interface between the A and C domain, and a calcium binding site of the parent alpha-amylase.
4. The method of claim 2, wherein the altered property is the substrate cleavage pattern and the structural part is located within 10 .ANG. from an amino acid residue of the substrate binding site.
5. A method of constructing a variant of a parent alpha-amylase having an altered property relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13 or has a sequence at least 70% homologous to said sequence when homology is determined by GAP using default values for the GAP program (Genetic Computer Group, Version 7.3) penalties, said method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ

ID NO:13 depicted in the Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) comparing the three-dimensional structure obtained in step (a) with a three-dimensional structure of an unrelated alpha-amylase, wherein the unrelated alpha-amylase differs from the parent alpha-amylase in said property;
(c) identifying a structural part of the three-dimensional structure obtained in step (a) which is different from the three-dimensional structure of the unrelated alpha-amylase and which is predicted to be relevant to said property;
(d) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and
(e) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,
wherein the variant has alpha-amylase activity and has one or more altered properties as compared to the parent alpha-amylase.
6. The method of claim 5, wherein, in step (d), the structural part of the parent alpha-amylase is modified so as to resemble the corresponding part of the unrelated alpha-amylase.
7. The method of clam 6, wherein, in step (d), the modification is a deletion, substitution, or insertion of one or more amino acid residues in the parent alpha-amylase so that the structural part in the variant corresponding to the structural part in the unrelated alpha amylase.
8. The method of claim 5, wherein the unrelated alpha-amylase is a fungal alpha-amylase or a mammalian alpha-amylase.
9. The method of claim 5, wherein the unrelated alpha-amylase is selected from the group consisting of: Aspergillus oryzae TAKA alpha-amylase, A. niger alpha-amylase, Bacillus subtilis alpha-amylase, and pig pancreatic alpha-amylase.
10. The method of claim 5, wherein the parent alpha-amylase is derived from a strain of Bacillus.
11. The method of claim 10, wherein Bacillus strain is selected from the group consisting of B. licheniformis, B. amyloliquefaciens, B. stearothermophilus, and an alkalophilic Bacillus sp.
12. The method of claim 11, wherein the parent alpha-amylase is derived from Bacillus strains NCIB 12289, NCIB 12512 or NCIB 12513.
13. The method of claim 5, wherein the structural part of the parent alpha-amylase identified in step (c) is selected from the group consisting of loop 1, loop 2, loop 3, and loop 8 of the parent alpha-amylase.
14. A method of constructing a variant of a parent alpha-amylase having a decreased Ca dependence of enzymatic activity or stability relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70% homologous to said sequence when homology is determined by the GAP program (Genetic Computer Group, Version 7.3) using default values for GAP penalties, said method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ ID NO:13 depicted in the Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) identifying in the structure obtained in step (a) one or more target amino acid residues within 10 .ANG. from a Ca.sup.2+ binding site;
(c) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said one or more target residues; and
(d) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,
wherein the variant has a decreased calcium ion dependency of enzymatic activity or stability as compared to the parent alpha-amylase.
15. A method of constructing a variant of a parent alpha-amylase having an altered pH dependence of enzymatic activity relative to the parent, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70% homologous to said sequence when homology is determined by the GAP program (Genetic Computer Group, Version 7.3) using default values for GAP penalties, said method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ ID NO:13 depicted in the Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) identifying in the three-dimensional structure obtained in step (a) a target amino acid residue within 15 .ANG. from an active site residue of the parent, wherein said target residue is predicted to be involved in electrostatic or hydrophobic interactions with said active site residue;

(c) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a substitution of said target residue with an amino acid which changes the electrostatic and/or hydrophobic surroundings of said active site residue; and
(d) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,
wherein the variant has alpha-amylase activity and has an altered pH dependence of enzymatic activity relative to the parent.
16. A method of constructing a variant of a parent alpha-amylase having increased thermostability and/or an altered temperature optimum of enzymatic activity relative to the parent alpha-amylase, wherein the parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70% homologous to said sequence when homology is determined by the GAP program (Genetic Computer Group, Version 7.3) using default values for GAP penalties, a method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ ID NO: 13 depicted in the Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a substitution at one or more position in the structure obtained in step (a), wherein said one or more positions correspond to a position in the three-dimensional structure depicted in the Appendix selected from the group consisting of L7, L61, Y62, F67, L75, K106, G145, I212, S151, R214, Y150, F143, R146, L241, I236, V259, F284, F350, F343, L427, V481, and wherein the amino acid residue at said position is replaced with a bulkier amino acid residue; and
(c) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,
wherein the variant has alpha-amylase activity and has increased thermostability and/or an altered temperature optimum of enzymatic activity relative to the parent alpha-amylase.
17. A method of constructing a variant of a parent alpha-amylase having an altered substrate cleavage pattern relative to the parent, wherein said parent alpha-amylase has the sequence of SEQ ID Nos: 2, 4, 6, or 13, or has a sequence at least 70% homologous to the sequence when homology is determined by the GAP program (Genetic Computer Group, Version 7.3) using default values for GAP penalties, said method comprising
(a) modelling the parent alpha-amylase on the three-dimensional structure of SEQ ID NO:13 depicted in the Appendix to produce a three-dimensional structure of the parent alpha-amylase;
(b) identifying in the structure obtained in step (a) a substrate binding area of the parent alpha-amylase;
(c) modifying the sequence of a nucleic acid encoding the parent alpha-amylase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said identified substrate binding area; and
(d) expressing said modified nucleic acid in a host cell to produce said variant alpha-amylase,
wherein the variant has a reduced ability to cleave a substrate close to the branching point.
18. The method of claim 17, wherein step (d) is achieved by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth.
19. The method of claim 14, wherein the parent alpha amylase has the sequence of SEQ ID NO:2 and the amino acid residue identified in step (b) corresponds to a position selected from the group consisting of: V102, I103, N104, H105, K106, R125, W155, W157, Y158, H159, F160, D161, G162, T163, Y175, K176, F177, G178, K180, A181, W182, D183, W184, E185, V186, S187, N192, Y193, D194, Y195, L196, M197, Y198, A199, D200, I201, D202, Y203, D204, H205, P206, V208, A209, D231, A232, V233, K234, H235, I236, K237, F238, F240, L241, A294, A295, S296, T297, Q298, G299, G300, G301, Y302, D303, M304, R305, K306, L307, W342, F343, L346, Q393, Y394, Y396, H405, H406, D407, I408, V409, R413, E414, G415, D416, S417, V419, A420, N421, S422, G423, L424, I428, T429, D430, G431, P432, V440, G441, R442, Q443, N444, A445, G446, E447, T448, W449, 1462, G475, Y480, V481, Q482, and R483.
20. The method of claim 15, wherein the parent alpha amylase has the sequence of SEQ ID NO:2 and the amino acid residue identified in step (b) corresponds to a position selected from the group consisting of E336, Q333, P331, I236, V102, A232, I103, L196, N326, H281, and Y273.

21. The method of claim 20, wherein the substitution in step (c) is selected from the group consisting of E336R, E336K, Q333R, Q333K, P331R, P331K, V102R, V102K, V102A, V102T, V102S, V102G, I236K, I236R, I236N, I103K, I103R, L196K, L196R, A232T, A232T, A232S, A232G, N326I, N326Y, N326F, N326L, N326V, H281F, H281I, H281L, Y273F, Y273W, and combinations of the foregoing.

22. The method of claim 17, wherein the parent alpha amylase has the sequence of SEQ ID NO:2 and the position modified in step (c) corresponds to a position selected from the group consisting of A52, V54, D53, Y56, Q333, and G57.

23. The method of claim 22, wherein the substitution in step (c) is selected from the group consisting of V54L, V54I, V54F, V54Y, V54W, V54R, V54K, V54H, V54E, V54Q, D53L, D53I, D53F, D53Y, D53W, Y56W, Q333W, A52W, A52Y A52L, A52F, A52I, and G57 substituted with any other amino acid.